APPENDIX A-1

Revised BrdU-ELISA Local Lymph Node Assay Protocol

Updated By the Japanese Center for the Validation of Alternative Methods (JaCVAM)
Validation Study Management Team

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Introduction

The LLNA: BrdU-ELISA is similar to the traditional LLNA in that it determines the sensitization potential of a test substance by measuring the proliferation of lymphocytes in the auricular lymph nodes draining the site of exposure (ears). One main difference is that the LLNA: BrdU-ELISA employs non-radioactive methods to assess lymphocyte proliferation by measuring bromodeoxyuridine (BrdU) uptake in the lymph nodes by an Enzyme Linked Immunosorbent Assay (ELISA) and thus foregoing the use of ^{3}H-methyl thymidine- or ^{125}I-iododeoxyuridine-based measurements used by the traditional LLNA.

This appendix describes the protocol used to obtain the BrdU-ELISA LLNA results (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a) evaluated in this technical summary of the validation status of the BrdU-ELISA LLNA. NICEATM requested a detailed BrdU-ELISA protocol, but it was not received prior to the release of the draft ICCVAM Background Review Document (BRD) on January 7, 2008. In the draft ICCVAM BRD, the protocol used by Takeyoshi et al. is contrasted with the ICCVAM recommended LLNA protocol (ICCVAM 1999). On February 27, 2008, the Japanese Center for the Validation of Alternative Methods (JaCVAM) provided revisions to the protocol so that it would accurately reflect the protocol approved by the International Validation Study Management for use in the interlaboratory validation studies. The protocol included herein reflects these changes.

Animal Selection and Preparation

Animal Species Selection

- Female CBA/JN mice were used.
- Mice should be 8-12 weeks old and their weight should not decrease during acclimatization. ICCVAM (1999) recommends mice be 8-12 weeks old and that weight variations between the mice should not exceed 20% of the mean weight.

Housing and Feeding Conditions

- Experimental animal room temperature should be 23 ± 2 °C. ICCVAM (1999) and Dean et al. (2001) recommend 21 ± 3 °C.
- Experimental animal room humidity should be 30% - 70% as recommended by ICCVAM (1999).
- Experimental animal rooms should be ventilated at 10-15 cycles per hour. ICCVAM (1999) makes no recommendation on ventilation.
- Experimental animal rooms should be lighted artificially for 12 hours per day.
- Mice should have free access to laboratory diet and drinking water.

Animal Preparation

- Mice should be acclimated for at least 5 days prior to the start of the test as recommended by ICCVAM (1999).

Control Substances

Solvent/Vehicle Control

- The solvents and vehicles control for all tests in the validation studies were acetone:olive oil (4:1 v/v), DMSO or Acetone.

Positive Control

- 50% (w/v) Hexyl cinnamic aldehyde was used as a concurrent positive control in the validation studies.

Test Procedure

Number of Animals per Dose

- Five mice per dose group and five mice for the vehicle control group should be used, as recommended by ICCVAM (1999). The initial studies performed by Takeyoshi et al. used four mice per dose group and four mice for the vehicle control group.

Selection of Doses

- At least three consecutive doses should be tested (e.g., 100%, 50%, 25%).
- Chemicals and doses used in the interlaboratory validation studies were determined by the Study Management Team in conjunction with the specialists in this field and considering the draft ICCVAM LLNA performance standards. All chemicals were coded by a biostatician. Dose selection was based on previous sensitization information such as dose range finding tests, guinea pig test results, or human potency. ICCVAM (1999) indicates that the highest dose tested should not induce systemic toxicity and/or excessive skin irritation.
- The Chief of Study Management Team served also as the chemical distributer.
- According to this coded list, the chemical distributer prepared the three doses of chemicals and positive control, and they were sent to each laboratory with adequate solvents one week prior to the start of testing. The samples were prepared, solubilized, suspepended or sonicated by each laboratory prior to the start of testing.

Dosing Schedule and Collection of Lymph Node Cells

- Days 1 through 3
  - Test substance or vehicle (25 ìL) was applied to the dorsum of each ear.
- Day 4
  - BrdU was intraperitoneally injected (5 mg/0.5mL/mouse). ICCVAM (1999) calls for no treatment on day 4.
- Day 5
  - The draining auricular lymph nodes were removed, weighed, and stored at -20°C until ELISA analysis. ICCVAM (1999) has no treatment on day 5. On day 6, ICCVAM (1999) advises an injection of 20 μCi ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine and 10^{-5}M fluorodeoxyuridine into the tail vein of each mouse and then removal of lymph nodes five hours later.

Observations

- The mice should be observed for clinical signs of local, excessive irritation or corrosion, or systemic toxicity as indicated ICCVAM (1999).

- In the internlaboratory validation studies, a commercial cell proliferation assay kit was used to quantify the incorporation of BrdU into lymph node cells (Boehringer Mannheim Corp., Indianapolis, IN, USA; Cat. No. 1647229).
- Lymph nodes were crushed, passed through a #70 nylon mesh and cells were suspended individually in 10-25 mL physiological saline. ICCVAM (1999) recommends using #200 stainless steel mesh.
- 100 μL cell suspension was added, in triplicate, to the wells of a flat bottom microplate.
- After centrifugation at 3000g for 10 minutes, the supernatants were removed, dried and 200 μL Fix-Denat was added to each well.
- The plate was allowed to stand at room temperature for 30 minutes, Fix-Denat was removed and the 100 μL diluted anti-BrdU antibody was added to each well.
- Cells were rinsed three times with phosphate buffered saline and then 100 μL substrate solution containing tetramethylbenzidine was added and allowed to react for 15 minutes at room temperature.
- Absorbance was read at 370 nm with a microplate reader with a reference wavelength of 492 nm or absorbance was read at 490 nm with a microplate reader with a reference wavelength of 650 nm in case of response stop solution (1M sulfuri acid). The absorbance was defined as the BrdU labeling index. The means absorbance of the vehicle control was around 0.05-0.3, which was used as an acceptance criteria. However, if possible, it was desirable to fall within the range of 0.1-0.2.
- The means and standard errors for the labeling index were calculated for each treatment group. The mean absorbances for the treatment groups were divided by the mean absorbance of the control group to determine the stimulation index (SI) for each treatment group. In case the positive control produced SI $\geq$ 2 as a success criteria, data of test substance at same time were accepted. If any concentration of test substance produced SI $\geq$ 2, the substance was identified as a sensitizer.
- For some tests, the concentration of a test substance required to produce SI=2 was derived by linear interpolation between the two concentration points above and below SI = 2.

Good Laboratory Practices (GLP)

The validation studies were not conducted under the full compliance of GLP. However, all the laboratories were GLP laboratories. In addition, all the laboratories that participated in the interlaboratory validation studies used the same experimental protocol and took part in a one day training seminar that explained the protocol and execution of the test method. Also, the same commercial kit, test materials and the same dose of each coded substance were used in all the laboratories. A formatted file for the entry of the experimental data and information was prepared using Microsoft EXCEL. The file was distributed to the experimental laboratories prior to the experiment. A preliminary test was performed with only a positive control substance to confirm that the experimental protocol was being adequately documented among the laboratories. After filling in the data, files from all the experimental laboratories were collected and the data were analyzed as

described in Omori et al. (2007).

After all testing, all records and documentation were checked by the Chief and biostatician on the Study Management Team. If questions regarding any of the data arose, all documents were requested from each laboratory.